Human Follicular Papilla Cells Carry Out Nonadipose Tissue Production of Leptin

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Leptin, a satiety-regulating cytokine, is predominantly expressed by adipocytes, although recently the nonadipose tissue production of leptin has been reported. To investigate the possibility of leptin production by human scalp hair follicles, we examined leptin production and its mRNA expression by cultured human follicular papilla cells. We isolated 12 human follicular papilla cell lines from different individuals. They were identified by their morphology, their high α-smooth-muscle actin expression, their inability to differentiate into adipocytes, and by the lack of mRNA for adipose-specific fatty acid binding protein. All the human follicular papilla cell lines, but not neonatal human dermal fibroblasts, produced significant amounts of leptin demonstrable by enzyme-linked immunosorbent assay. We demonstrated leptin mRNA expression by human follicular papilla cell lines, but not by neonatal human dermal fibroblasts, by reverse transcription polymerase chain reaction. By immunohistochemistry and *in situ* hybridization, we detected both leptin protein and mRNA at the lower portion of the hair follicle, i.e., hair matrix, inner root sheath of the hair bulb, and human follicular papilla cells. In contrast, the leptin receptor with intracytoplasmic signal sequence was detected in the follicular papilla cells immunohistochemically, and the long isoform of the leptin receptor mRNA was demonstrated in the human follicular papilla cell lines by reverse transcription polymerase chain reaction. Finally, by using these human follicular papilla cell lines, we showed that cytokines such as interleukin-1β, tumor necrosis factor α, interferon-γ, and interleukin-4, and growth factors such as epidermal growth factor, basic fibroblast growth factor, and transforming growth factor β1, but not vascular endothelial growth factor, hepatocyte growth factor, keratinocyte growth factor, and insulin-like growth factor 1, significantly downregulated the production of leptin. These data demonstrated that human follicular papilla cells produce leptin and express the functional leptin receptor *in vivo* and *in vitro*, suggesting its autocrine function. Moreover, the regulation pattern of its production by various factors suggests a pivotal role of leptin in hair biology. ***Key words: adipocytes/follicular papilla cell/hair/leptin. J Invest Dermatol 117:1349–1356, 2001***

Leptin, the ob gene product, has been characterized as a satiety-regulating cytokine that is predominantly expressed by adipocytes and secreted into the blood stream (Zhang *et al*, 1994; Maffei *et al*, 1995; Masuzaki *et al*, 1995). Obese/obese (ob/ob) mice, homozygous for a spontaneous mutation in the ob gene, fail to produce leptin and exhibit severe obesity. The appetite-regulating effect of leptin has been shown to be dependent on the binding of leptin to the corresponding leptin receptor subtype ObRb in the hypothalamic region (Halaas *et al*, 1995; Ghilardi *et al*, 1996). The leptin receptor splice variant ObRb was shown to be expressed in various tissues in rodents, however, including lung, pancreatic β islets, and kidney (Fei *et al*, 1997), suggesting that leptin additionally mediates extrahypothalamic actions and thus might exert diverse biologic functions (Hoggard *et al*, 1997; Lollmann *et al*, 1997). Subsequently, it has been reported that leptin triggers reproductive functions *in vivo* (Chehab *et al*, 1997) and serves as a mitogen for a growing number of cell types, including endothelial cells, monocytes, lung epithelial cells, and pancreatic β cells *in vitro* (Tanabe *et al*, 1997; Bouloumie *et al*, 1998; Santos-Alvarez *et al*, 1999; Tsuchiya *et al*, 1999). In addition, leptin has been suggested to play an important role in angiogenesis, because leptin promotes the formation of new blood vessels (Bouloumie *et al*, 1998; Sierra-Honigmann *et al*, 1998).

Although leptin was initially reported to be produced predominantly by adipocytes, several investigators have demonstrated its nonadipose tissue production. Masuzaki *et al* (1997) have reported leptin production by human placental trophoblasts and amnion cells from the uteri of pregnant women. Furthermore, Hoggard *et al* (1997) have revealed the presence of leptin mRNA in fetal cartilage/bone and hair follicles as well as the placenta. In spite of these studies, the following questions still remain unanswered: what cells in the follicular structure produce leptin, and is leptin also produced in human scalp hair? Therefore, in this study, we examined leptin production and its mRNA expression by cultured human follicular papilla cells (FPC). We found that more than 10 FPC lines from the scalp of three different donors secreted significant amounts of leptin *in vitro* and expressed mRNA for leptin. Furthermore, both our *in situ* hybridization for leptin

Manuscript received Aptil 1, 2001; revised August 27, 2001; accepted for publication September 10, 2001.

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Abbreviations: FPC, follicular papilla cell; HGF, hepatocyte growth factor; KGF, keratinocyte growth factor; NHDF, neonatal human dermal fibroblast; VEGF, vascular endothelial growth factor.

mRNA and immunohistochemical staining for leptin protein demonstrated positive signals in FPC, although the positive signals were also recognized in the hair matrix and inner root sheath of the hair bulb. On the other hand, we demonstrated the expression of the functional leptin receptor in the FPC *in vivo* and *in vitro*. In addition, using these FPC, we could show the regulation of leptin production by FPC by various cytokines or growth factors *in vitro*.

MATERIALS AND METHODS

Media and reagents The medium used in the study was Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin, streptomycin, and fungizone antibiotic solution (Sigma, St. Louis, MO), and 10% fetal bovine serum (Bioserum, Canterbury, Victoria, Australia) (complete medium). We used the following cytokines and growth factors: interleukin-1β (IL-1β), interleukin-4 (IL-4), tumor necrosis factor α (TNF-α), interferon-α (IFN-α), interferon-γ (IFN-γ), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), transforming growth factor β1 (TGF-β1), and insulin-like growth factor-1 (IGF-1) (PeproTech, Rock Hill, NJ).

Cell cultures Normal skin was obtained from three patients with benign scalp tumors. We used the safety margins included for the surgical excision of tumors on the scalp of a 33-y-old-female, 42-y-old female, and 56-y-old male. After removing the dermal sheets, the hair bulbs were placed on culture dishes. FPC, isolated as described by Messenger (1984), were grown to subconfluence in complete medium and used in passages 4–6. By these procedures, we obtained different FPC lines (3301, 3302, 3306, 0901, 0904, 0905, 0906, 1201, 1203, 1204, 1205, 1206), and FPC clone 1206a by a limiting dilution method from the parent cell line 1206. As a control, we used two different lines of neonatal human dermal fibroblasts (HNDF), which we designated NHDF-1 and NHDF-2, purchased from Kurabo, Osaka, Japan. NHDF-1 and NHDF-2 were grown in complete medium and used in passages 6–8. Cells were cultured at 37°C in a humidified atmosphere containing 10% CO_2. To measure leptin production, we cultured these FPC lines, clone, or HNDF for various time periods, and the culture supernatants were recovered from these cultures. In some experiments, FPC lines 3301, 3302, and 3306 were stimulated with various concentrations of IL-1β (100 ng per ml, 10 ng per ml, 1 ng per ml), IL-4 (100 ng per ml, 10 ng per ml, 1 ng per ml), TNF-α (100 ng per ml, 10 ng per ml, 1 ng per ml), IFN-α (1000 U per ml, 100 U per ml, 10 U per ml), IFN-γ (1000 U per ml, 100 U per ml, 10 U per ml), EGF (1000 ng per ml, 100 ng per ml, 10 ng per ml), bFGF (100 ng per ml, 10 ng per ml, 1 ng per ml), VEGF (1000 ng per ml, 100 ng per ml, 10 ng per ml), HGF (100 ng per ml, 10 ng per ml, 1 ng per ml), KGF (100 ng per ml, 10 ng per ml, 1 ng per ml), TGF-β1 (100 ng per ml, 10 ng per ml, 1 ng per ml), and IGF-1 (1000 ng per ml, 100 ng per ml, 10 ng per ml) for 72 h. To measure the concentration of leptin in the culture supernatants, all the cultures were performed in triplicate. To examine leptin mRNA in cultured cells, an FPC line or NHDF-1 was cultured on Lab-Tek chamber slides (Nunc, Naperville, IL).

We also cultured rat preadipocytes purchased from Sangi (Hokkaido, Japan), and the stromal cell fraction of human adipose tissue isolated by collagenase digestion according to Hauner *et al* (1989). These cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 17 μM pantothenate, 33 μM biotin, 100 μM ascorbic acid, and 50 nM triiodothyronine (growth medium). To induce their differentiation into mature adipocytes, they were refed with growth medium supplemented with 10 μg per ml insulin and 2.5 μM dexamethasone (adipocyte differentiation medium). At the same time, some FPC lines were also cultured with differentiation medium. After 16 d, the culture was observed under an inverted microscope and cells were defined as adipocytes that differentiated *in vitro* when their cytoplasm was completely filled with small or large lipid droplets.

Immunoblotting Two FPC lines and two HNDF were washed twice in cold phosphate-buffered saline and resuspended in 100 μl of lysis buffer [1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM ethylenediamine tetraacetic acid (EDTA), 10 mg per ml leupeptin, 10 mg per ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate]. The nuclei and the insoluble cell debris were removed by centrifugation at 4°C for 10 min at 14,000*g*. The postnuclear extracts were collected and used as total cell lysates. Total cell lysates were suspended in 2 × sodium dodecyl sulfate (SDS) sample buffer [313 mM Tris-HCl (pH 6.8), 10% SDS, 2-mercaptoethanol, 50% glycerol, and 0.01% bromophenol blue] and heated at 95°C for 3 min. The protein samples were fractionated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was stained with an avidin–biotin alkaline phosphatase technique (ImmunoStar Kit, Wako Pure Chemical Industries, Osaka, Japan) using mouse monoclonal anti-α-smooth-muscle actin antibody (Enzo Diagnostic, Farmingale, NY) or mouse monoclonal anti-β-actin antibody (Sigma). Briefly the membrane was first blocked with rabbit serum (diluted 1:10) for 30 min and then exposed to primary antibodies at 4°C overnight. The membrane was further treated by biotin-conjugated antimouse IgG (H + L) at 37°C for 20 min, and then incubated with streptavidin–biotin labeled horseradish peroxidase complex at 37°C for 20 min. Finally, the blots were visualized by chemiluminescence.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) Total RNA was extracted from FPC lines (1201, 1204, and 0904), a clone, 1206a, and NHDF-1 and NHDF-2 using the guanidinium thiocyanate method described by the manufacturer (ISOGEN; Nippon Gene, Toyama, Japan), and RNA was quantified by spectrophotometry. We also used RNA from human cutaneous adipose tissue (BioChain Institute, Hayward, CA) as a control. First-strand cDNA was synthesized from total RNA extracted in RNAse-free conditions. cDNA was obtained from total RNA using TaKaRa RNA PCR kit (AMV) (Takara Biochemicals, Osaka, Japan), as described in the manufacturer's protocol. PCR was performed in a Perkin Elmer 2400 thermal cycler in a final volume of 20 μl reaction mixture containing 1 μM of each forward and reverse primer. The primer sets for leptin, human adipose-specific fatty acid binding protein aP2 (haP2), human leptin receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed to span exon–intron boundaries as +46/leptin 5′-CTTTTCTATGTCCAAGCTGTGCCC-3′ forward primer and –350/leptin 5′-CAGCTCTTAGAGAAGGCCAGCACG-3′ reverse primer, +2/haP2 5′-CAGCTTCCTTCTCACCTGAAGA-3′ forward primer and –463/haP2 5′-CTGGACTGAAGTTCGCATTGAAC-3′, +2810/leptin receptor 5′-GAAGATGTTCCGAACCCCAAGAAT-3′ forward primer and –3237/leptin receptor 5′-CTAGAGAAGCACTT-GGTGACTGAA-3′ reverse primer, +66/GAPDH 5′-GAAGGTFAAFF-TCGGAGTC-3′ forward primer and –291/GAPDH 5′-GAAGATGG-TGATGGGATTC-3′ reverse primer, and +51/GAPDH 5′-TGAA-GGTCGGAGTCAACGCATTTGGT-3′ forward primer and –1053/G3PDH 5′-CATGTGGGCCATGAGGTCCACCAC-3′ reverse primer, respectively. The reaction mixture was subjected to 30 cycles of PCR with the following conditions: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. PCR products were visualized on 1.2% agarose gels containing 0.1 μg per ml ethidium bromide. PCR products amplified using the primer sets for the leptin receptor were digested by EcoRV (Takara Biotechnology, Tokyo, Japan).

Direct sequencing The RT-PCR products were purified by using glasspowder (EASYTRAP Ver.2, Takara Biochemicals). The PCR products were sequenced by both forward and reverse primers using a BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer Biosystems, Warrington, U.K.). The DNA sequence was collected and analyzed on an ABI Prism 377 DNA sequencer (Perkin-Elmer).

Immunohistochemistry The paraffin-embedded 5 μm tissue sections of human scalp skin from three different donors were stained with an avidin–biotin alkaline phosphatase technique using monoclonal antihuman leptin antibody (10 μg per ml) (Genzyme/Techne, Cambridge, MA), isotype-matched control antibody (10 μg per ml) (PharMingen, San Diego, CA), polyclonal rabbit antihuman leptin antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), or nonimmune rabbit serum (1:200 dilution, Sigma). We also used monoclonal antimouse leptin receptor antibody (10 μg per ml) (Santa Cruz Biotechnology), which recognizes the epitope mapping at the carboxy terminus of the mouse leptin receptor and cross-reacts with the human leptin receptor. Briefly, following the treatment with 0.1% trypsin in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.1% $CaCl_2$ for 15 min at 37°C, rehydrated sections (5 μm thick) were first blocked with rabbit serum for mouse antibody or goat serum (diluted 1:10) for rabbit antibody for 30 min, and then exposed to primary antibodies at 4°C overnight. Antibody binding was demonstrated via avidin–biotin alkaline phosphatase staining using Histofine SAB-AP(M) kit or Histofine SAB-AP(R) kit, Nichirei, Tokyo, depending on the primary antibodies. The specificity of the immunoreaction by antileptin antibody

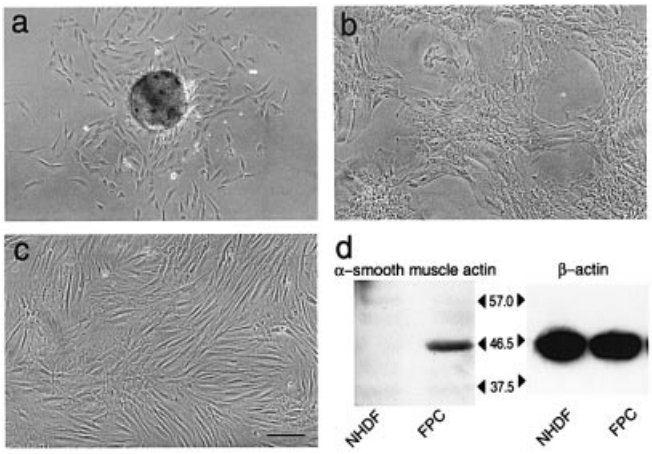

Figure 1. A representative FPC line, 1206, demonstrated the characteristic morphology and higher expression of α-smooth-muscle actin than neonatal human dermal fibroblasts. An FPC line was isolated as described by Messenger (*a*), and was grown to subconfluence in the complete medium (*b*). As a control, we cultured NHDF (*c*). Total cell lysates of the FPC line and NHDF were fractionated by 12% SDS-PAGE and transferred onto PVDF membrane. The membrane was immunoblotted using anti-α-smooth-muscle actin antibody or anti-β–actin antibody (*d*). *Scale bar*: 200 μm.

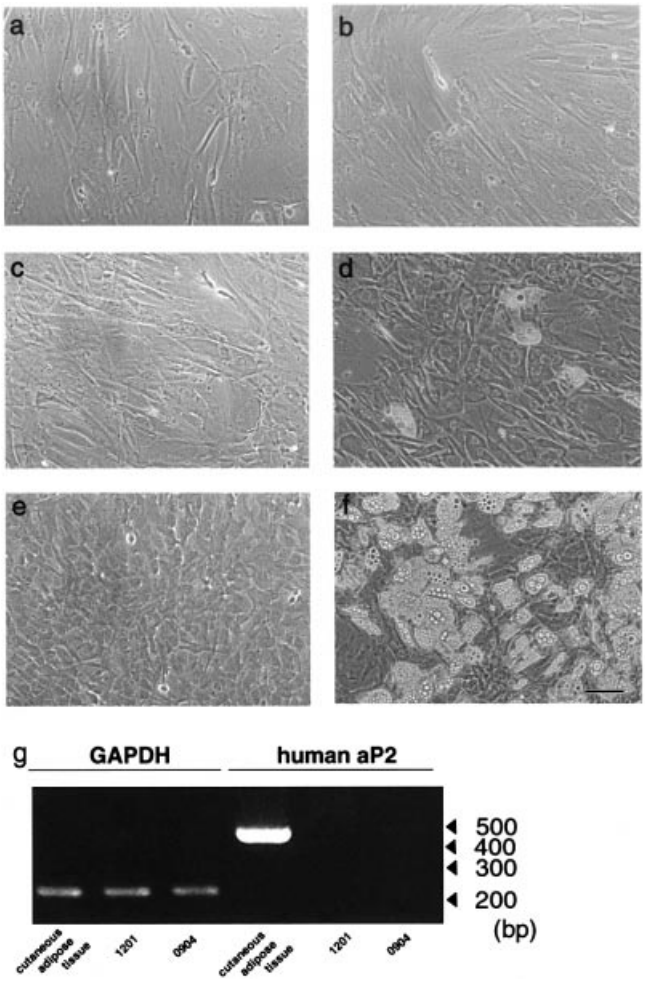

Figure 2. FPC lines did not differentiate into adipocytes. We cultured FPC (*a*), the stromal cell fraction of the human adipose tissue isolated by collagenase digestion (*c*), and rat preadipocytes (*e*). Rat preadipocytes and the stromal cell fraction of the human adipose tissue were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 17 μM pantothenate, 33 μM biotin, 100 μM ascorbic acid, and 50 nM triiodothyronine (growth medium). To induce their differentiation into mature adipocytes, they were refed with growth medium supplemented with 10 μg per ml insulin and 2.5 μM dexamethasone (adipocyte differentiation medium). At the same time, FPC were also cultured with adipocyte differentiation medium. After 16 d, the culture was observed under an inverted microscope: (*b*) FPC; (*d*) stromal cells; (*f*) rat preadipocytes. Total RNA from cutaneous adipose tissue, two FPC lines, 0904 and 1201, and NHDF was subjected to reverse transcription followed by PCR amplification using the primer sets for GAPDH and human aP2, respectively. The reaction mixture was subjected to 30 cycles of PCR with the following conditions: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. PCR products were visualized on 1.2% agarose gels containing 0.1 μg per ml ethidium bromide (*g*). *Scale bar*: 200 μm.

was further confirmed by preabsorption of antileptin antibody with recombinant leptin (Sigma).

Enzyme-linked immunosorbent assay (ELISA) for leptin production The culture supernatants of the FPC lines, clone, and NHDF lines were recovered at various time points after the start of the culture. In other experiments, the culture supernatants of FPC lines with or without the stimulation of cytokines or growth factors were recovered 72 h after culture. The production of leptin was measured by ELISA using a human leptin immunoassay kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The levels of leptin were calculated by using a standard curve obtained with recombinant leptin (from 0 to 1000 pg per ml). When the culture supernatants contained leptin over the upper limit of the standard curve, they were measured again after optimal dilution. The data are expressed as the mean of triplicate cultures.

Preparation of riboprobes The plasmid template for the riboprobe consisted of the 304 bp PCR product of human leptin, which was synthesized by PCR amplification and confirmed by DNA sequencing as described above. This PCR product was cloned into a pGEM-T Easy Vector using pGEM-T Easy Vector systems (Promega, Madison, WI). Aliquots of the plasmids enclosing leptin cDNA were linearized with PstI and SacII restriction enzymes. Digoxigenin-labeled complementary RNAs (cRNAs) were prepared *in vitro* by using the DIG RNA labeling kit (Roche Diagnostics, Basel, Switzerland). T7 and Sp6 RNA polymerases were used to produce sense and antisense probes for leptin according to the kit protocol. The amount of cRNA obtained was quantified using serial dilutions from 0.25 pmol per μl to 0.25 fmol per μl. The dilutions were spotted onto a positively charged nylon membrane (Roche Diagnostics) and cross-linked using a UV Stratalinker (model 1800; Stratagene, La Jolla, CA). Spots were detected by incubation in freshly prepared NBT/X phosphate color development solution (Roche Diagnostics) for 2 h in the dark. The yield of DIG-labeled probe was determined by comparison to a DIG-labeled control.

***In situ* hybridization** *In situ* hybridization using the DIG-labeled probes was performed as described previously (Miller *et al*, 1993; Panoskaltsis-Mortari and Bucy, 1995). All solutions were treated with diethyl pyrocarbonate and all glassware was baked at 240°C overnight to prevent RNA degradation. Five micron cryostat sections of human scalp skin on silane-coated slides or the Lab-Tek chamber slides on which an FPC line or NHDF-1 were cultured, were fixed for 20 min in 4% paraformaldehyde. The slides were rinsed in 2 × sodium citrate/chloride buffer (SSC), treated with proteinase K (1 μg per ml in 0.1 M Tris, 50 mM EDTA, 20 min, 37°C), and acetylated for 10 min in 0.1 M triethanolamine (pH 8.0), 0.9% sodium chloride, and 0.25% acetic anhydride. After the slides were dipped once in 2 × SSC, dehydrated in ascending series of ethyl alcohol, and air-dried, they were incubated overnight at 50°C in a humidified chamber with the hybridization buffer (mRNA *in situ* hybridization solution, Dakopatts, Denmark) containing freshly denatured cRNA probes at 50 ng per section. The posthybridization washes were with 50% formamide in 2 × SSC for 20 min at 50°C (twice). Then the slides were washed with 500 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 8) (NTE) for 10 min at 37°C, followed by incubation with RNAse A (20 μg per ml, Roche Diagnostics) in NTE at 37°C for 30 min to remove the nonhybridized RNA. The slides were washed with NTE for 5 min at 37°C, in 2 × SSC for 20 min at 50°C (once), in 0.2 × SSC for 20 min at 50°C (once), in 0.1 × SSC for 20 min at 50°C (once), and in 100 mM Tris-

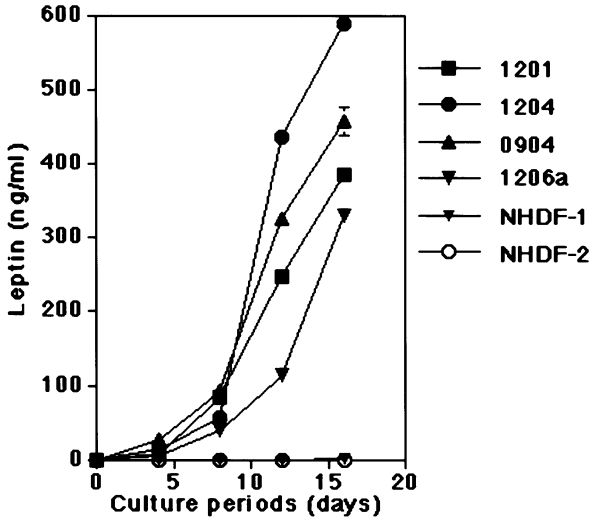

Figure 3. FPC lines produced leptin. Three different FPC lines, 1201, 1204, 0904, and a clone, 1206a, and NHDF lines NHDF-1 and NHDF-2 were cultured for 20 d, and the culture supernatants were recovered at various time points after the start of culture. The amount of leptin in the culture supernatants was measured by ELISA. Representative data of three different experiments are shown.

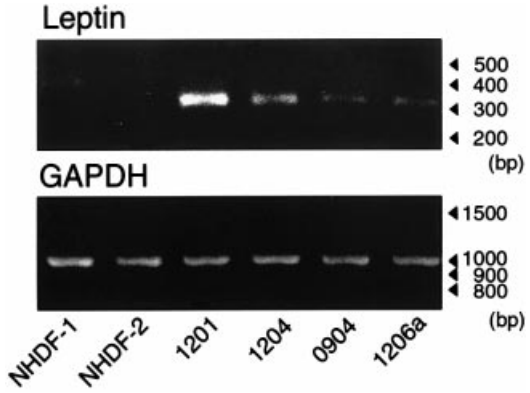

Figure 4. FPC lines expressed leptin mRNA. Total RNA was extracted from three FPC lines, 1201, 1204, 0904, and a clone, 1206a, and the two NHDF lines using the guanidinium thiocyanate method, and then cDNA was reverse-transcribed from total RNA. PCR was performed using the primer sets for leptin and GAPDH, respectively. The reaction mixture was subjected to 30 cycles of PCR with the following conditions: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. PCR products were visualized on 1.2% agarose gels containing 0.1 μg per ml ethidium bromide.

HCl (pH 7.5) for 5 min at room temperature. Signals were detected by incubating in 1 × blocking solution (Roche Diagnostics) at room temperature for 60 min followed by incubation with anti-DIG alkaline phosphatase antibody (1:100; Dakopatts) in 1 × blocking solution for 1 h at room temperature. The slides were washed in 100 mM Tris (pH 8), 150 mM sodium chloride (pH 7.5) twice, in color detection buffer (Roche Diagnostics) for 10 min each, and then incubated in freshly prepared NBT/X phosphate color development solution (Roche Diagnostics) overnight in a light-sealed humidified chamber at room temperature. The reaction was stopped by washing the slides in TE buffer, pH 8.0; they were then coverslipped with permafluor (Lipshaw Immunon, Pittsburgh, PA).

RESULTS

Culture of FPC We isolated 12 FPC lines and one clone from three different donors. All these cells exhibited a polygonal and aggregative morphology in sparse and subconfluent states, respectively, and did not contain any lipid droplets. When we examined for α-smooth-muscle actin expression in some of the FPC lines, they showed a significantly high expression. **Figure 1** shows representative data in one of the FPC lines, 1206, and those of NHDF-1. To exclude the possibility of contamination of the FPC lines with preadipocytes or adipocytes, we cultured some of the FPC lines in adipocyte differentiation medium for 16 d. During that culture period, none of these FPC lines was induced to store lipid droplets (**Fig 2*b***). In contrast, most of the rat preadipocytes and about 10% of the stromal cell fraction of the human adipose tissue differentiated into adipocytes in the same culture medium (**Fig 2*c*–*f***). In addition, RT-PCR analysis demonstrated that FPC lines 1201 and 0904 lacked mRNA for haP2, whereas total RNA extracted from human cutaneous adipose tissue as a positive control contained its mRNA (**Fig 2*g***).

Demonstration of leptin production by FPC lines and clone and their expression of leptin mRNA To examine leptin production by human hair follicles, at first we determined whether the FPC lines and clone produced leptin or not. For this purpose, we cultured three different FPC lines, 1201, 1204, and 0904, and a clone, 1206a, and NHDF-1 and NHDF-2 for 20 d, and then recovered the culture supernatants at various time points after the start of the culture to analyze for leptin by ELISA. **Figure 3** shows representative data of three different experiments, demonstrating that all the FPC lines and the clone produced leptin in a time-dependent fashion, whereas none of the NHDF lines produced detectable levels of leptin. Next, we examined whether these cell lines and clone expressed leptin mRNA. Again, all the FPC lines and clone, but not HNDF, expressed a PCR product approximately 304 bp in length (**Fig 4**). Thus, we directly sequenced these PCR products. The sequence analysis revealed that the sequences of amplified cDNA from three FPC lines and the clone exactly matched the reported leptin sequence.

Immunohistologic demonstration of leptin protein in the scalp skin Next, we examined the expression pattern of leptin protein in normal human scalp skin by immunohistochemistry using antileptin antibody. **Figure 5** shows representative data of three different examinations using scalp skin from different donors. Positive leptin staining was recognized only at the lower portion of the hair follicle and in scattered adipocytes (**Fig 5*a***). No positive staining was observed on any layers of the epidermis, any other portions of the hair follicles above the hair bulb, eccrine sweat glands, sebaceous glands, or any dermal cell components. Examination of the hair bulb area at a higher magnification revealed that the hair matrix, inner root sheath of the hair bulb, and FPC were positively stained with antileptin antibody (**Fig 5*c***). A similar staining pattern was obtained with either polyclonal antileptin antibody (**Fig 5*a*, *c***) or monoclonal antileptin antibody (**Fig 5*e***). No positive staining was shown with either nonimmune rabbit serum (**Fig 5*b***), isotype-matched control antibody (**Fig 5*f***), or antileptin antibody that was preabsorbed by leptin (**Fig 5*d***).

In situ* hybridization for leptin mRNA expression in scalp skin** When we examined the expression of leptin mRNA in scalp skin with the *in situ* mRNA hybridization technique, hybridization signals with the antisense probe to leptin were detected in the hair bulb area including the hair matrix and inner root sheath of the hair bulb, and FPC (**Fig 6*b*, *d), whereas no hybridization signals were recognized with the sense probe to leptin (**Fig 6*a*, *c***). In addition, positive hybridization signals were detected on cultured FPC lines only by the antisense probe (**Fig 6*e*, *f***). These data were consistent with those of the above-described immunohistochemical studies.

The expression of leptin receptor mRNA by FPC lines and immunohistologic demonstration of leptin receptor in scalp skin Next, we examined the expression of the leptin receptor in normal human scalp skin by immunohistochemistry using antileptin receptor antibody. It is well known that there are several variants of the leptin receptor as a result of alternative splicing of a single

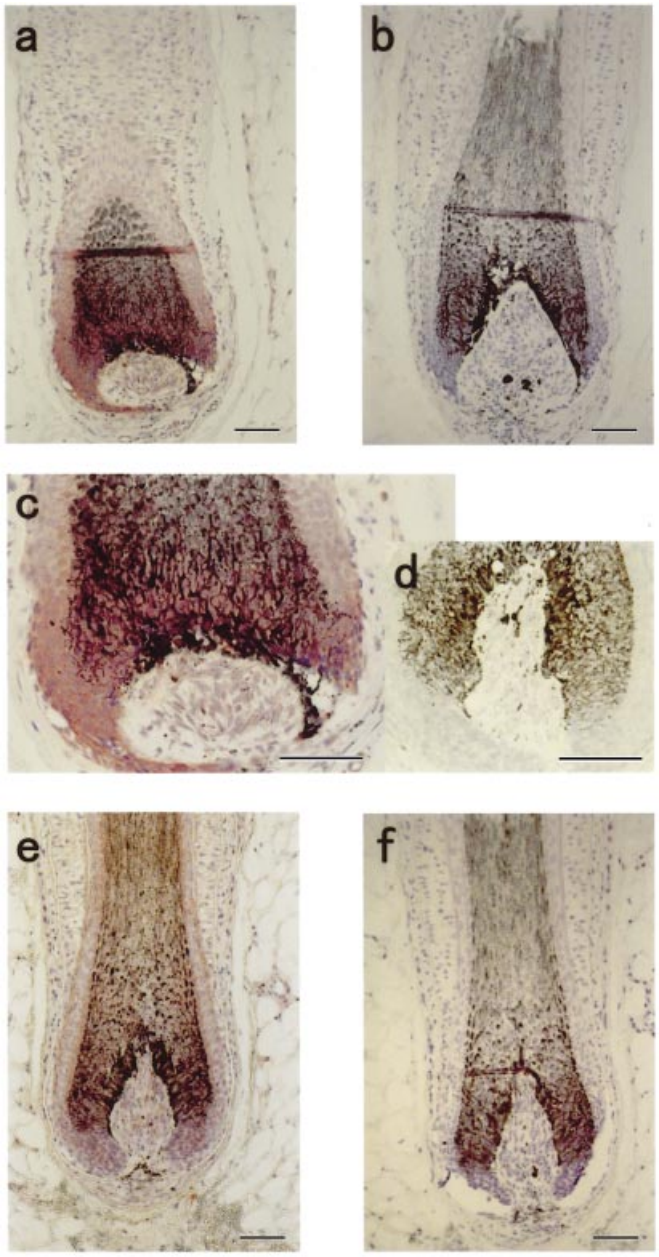

Figure 5. Leptin protein was detected at the lower portion in the hair follicle. After tryptic digestion of paraffin-embedded 5 μm tissue sections, the sections were stained with an avidin–biotin alkaline phosphatase technique using polyclonal rabbit antihuman leptin antibody (1:200 dilution), nonimmune rabbit serum (1:200 dilution), monoclonal antihuman leptin antibody (10 μg per ml), or isotype control antibody (10 μg per ml). The sections were also stained by the same procedure replacing antileptin antibody with that preabsorbed with leptin. *Scale bar*: 100 μm.

transcript encoded by the leptin gene (Tartaglia *et al*, 1995; Lee *et al*, 1996). Among them, only its long isoform contains an approximately 300-amino-acid intracellular domain and leads to activation of the JAK (Janus kinase) and STAT (signal transducer and activator of transcription) cascades (Ghilardi *et al*, 1996; Vaisse *et al*, 1996; White and Tartaglia, 1996). **Figure 7** shows representative data from three different examinations of scalp skin from different donors by using monoclonal antileptin receptor antibody. The antibody we used in this study recognizes the carboxy-terminal cytoplasmic domain, which is found only in the long isoform of the leptin receptor (Funahashi *et al*, 2000). By using it, positive leptin receptor staining was recognized in the FPC, scattered spindle cells in the perifollicular fibrous tissue, adipocytes, fibroblasts, and some lymphoid cells infiltrating in the dermis. No positive staining was observed on any layers of the epidermis or any other portions of the hair follicles above the hair bulb (**Fig 7*a***, ***b***). An isotype control antibody produced no positive signal (**Fig 7*c***). Consistent with these immunohistologic studies, the results of RT-PCR using the primer sets for the long isoforms of the leptin receptor (Shimon *et al*, 1998) revealed the presence of the intracellular signaling domain of the human lepin receptor in adipose tissue, FPC lines, and NHDF (428 bp DNA products; **Fig 7*d***). The specificity of the expressed band was confirmed by incubation with EcoRV, appropriately digesting the region to the expected 280 and 148 bp products (**Fig 7*e***).

Regulation of leptin production with cytokines or growth factors So far, we do not have any information about the role played by leptin produced by FPC in hair growth. To address this issue, we examined the effects of various cytokines or growth factors that are suggested to affect hair growth. We added IL-1β, IFN-α, and TNF-α as representatives of proinflammatory cytokines, IFN-γ and IL-4 as Th1 and Th2 cytokines, respectively, EGF, bFGF, and TGF-β1 as growth factors that retard hair growth (Moore *et al*, 1983; du Cros, 1993; Sellheyer *et al*, 1993; Philpott *et al*, 1994), and VEGF, HGF, KGF, and IGF-1 as growth factors that augment hair growth (Philpott *et al*, 1994; Danilenko *et al*, 1995; Shimaoka *et al*, 1995; Guo *et al*, 1996; Lachgar *et al*, 1996; Bol *et al*, 1997). **Figure 8** shows representative data of three different experiments using FPC line 3306. Similar data were also obtained in the experiments using FPC lines 3301 and 3302. All the inflammatory cytokines except for IFN-α and the growth factors that suppress hair growth significantly downregulated the production of leptin, whereas those that support hair growth did not affect its production.

DISCUSSION

In murine fetus, it was reported that leptin and its receptor mRNA was expressed at the lower portion of the hair follicle (Hoggard *et al*, 1997). Our study using *in situ* hybridization for leptin mRNA and immunohistochemical staining for leptin protein further demonstrated that even in adult human scalp hair both leptin mRNA and protein were expressed at the lower portion of the hair follicle, i.e., in the hair matrix, inner root sheath of the hair bulb, and FPC. Furthermore, the production of leptin by FPC could be detected in the culture supernatants of the cultured FPC lines. In addition, the expression of the functional leptin receptor was detected in the FPC immunohistologically, which was confirmed by the demonstration of mRNA for the long isoform of the leptin receptor.

In this study, we isolated FPC by a standard procedure reported by Messenger (1984). These FPC demonstrated the characteristics of FPC described previously, such as the aggregative morphology at a subconfluent state and a significantly high α-smooth-muscle actin expression. In spite of these data, our findings that FPC produced significant amounts of leptin pose the questions of whether FPC isolated by this procedure were contaminated by preadipocytes surrounding the hair bulbs and whether FPC are specialized preadipocytes aggregated in the hair bulb. Therefore, we cultured FPC in adipocyte differentiation medium. In contrast to human and rat preadipocytes, FPC did not differentiate into adipocytes in the adipocyte differentiation medium during 3 wk. Furthermore, our FPC lacked mRNA for human aP2, which could definitely exclude the possibility of contamination with adipocytes in our FPC. In addition, recently, Bornstein *et al* (2000) reported that cultured preadipocytes did not produce any detectable leptin in culture supernatants before 5 d incubation in adipocyte differentiation medium. In contrast, our FPC produced a significant amount of leptin even in the ordinary culture medium that did not contain any chemicals required for adipocyte differentiation. These data not only ruled out the possibility of contamination with

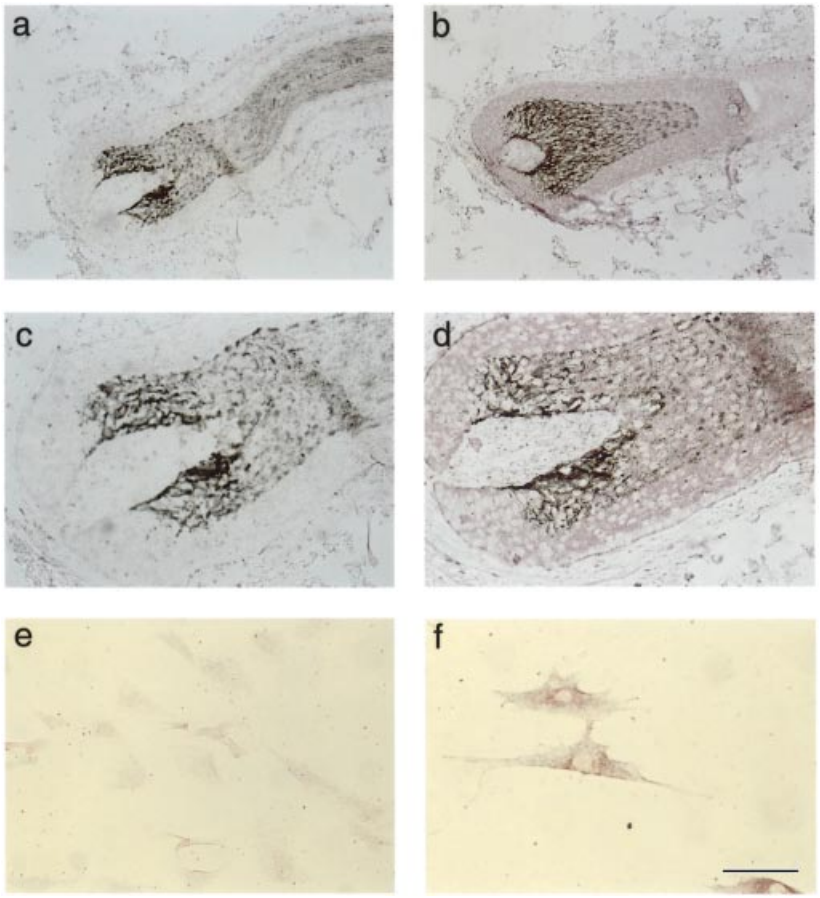

Figure 6. Leptin mRNA was detected at the lower portion in the hair follicle and in a cultured FPC line. *In situ* hybridization using the DIG-labeled probes was performed on a 5 μm cryostat section. The tissue sections (*a*, *b*, *c*, *d*) and the cultured cells (*e*, *f*), which were fixed in 4% paraformaldehyde and prehybridized, were incubated overnight at 37°C in a humidified chamber with the prehybridization solution containing freshly denatured cRNA probes at 50 ng per section. After posthybridization washes, these preparations were reacted with anti-DIG alkaline phosphatase antibody (1:500) and the signals were revealed by freshly prepared NBT/X-phosphate color development solution. Antisense probe to leptin, and sense probe. *Scale bar*: (*a*, *b*) 200 μm; (*c–f*) 100 μm.

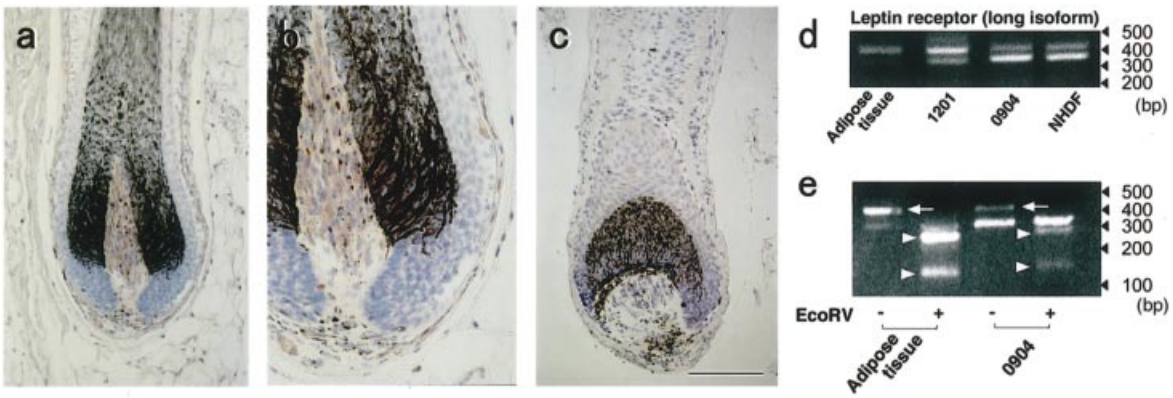

Figure 7. The functional leptin receptor was expressed in the FPC. After tryptic digestion of paraffin-embedded 5 μm tissue sections, the sections were stained with an avidin–biotin alkaline phosphatase technique using monoclonal antileptin receptor antibody (10 μg per ml), which recognizes the carboxy-terminal cytoplasmic domain, or isotype control antibody (10 μg per ml). Total RNA from cutaneous adipose tissue, two FPC lines, 0904 and 1201, and NHDF was subjected to reverse transcription followed by PCR amplification using the primer sets for the long isoform of the leptin receptor. The reaction mixture was subjected to 30 cycles of PCR with the following conditions: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. PCR products were visualized on 1.2% agarose gels containing 0.1 μg per ml ethidium bromide (*d*). To confirm the specificity of the expressed band (*arrows*), PCR products were treated with EcoRV (*e*). The *arrowheads* indicate the expected digested bands corresponding to 280 and 148 bp products. *Scale bar*: 100 μm. *Scale bar*: (*a*, *c*) 200 μm; (*b*) 100 μm.

preadipocytes in our cultured FPC, but further suggest that FPC are a unique population of cells with the ability to produce leptin distinct from preadipocytes or adipocytes.

Our demonstration of the production of leptin by FPC suggest a significant role played by leptin in hair biology. In addition, we found the expression of the functional leptin receptor on the FPC.

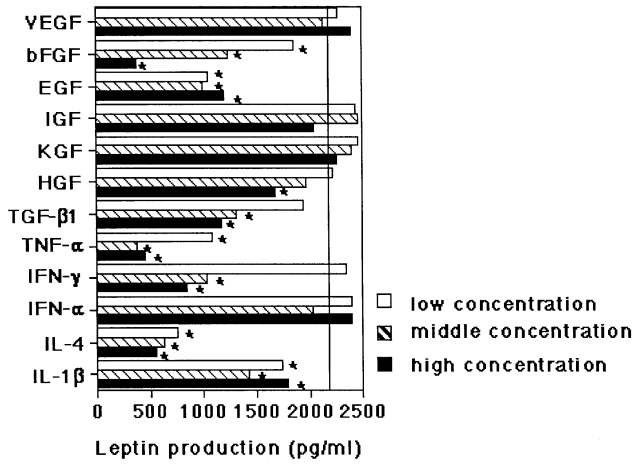

Figure 8. Leptin production by FPC was regulated differently by various cytokines and growth factors. To examine the effects of various cytokines or growth factors that are suggested to affect hair growth, an FPC line, 3306, was stimulated with various concentrations of IL-1β (100 ng per ml, 10 ng per ml, 1 ng per ml), IL-4 (100 ng per ml, 10 ng per ml, 1 ng per ml), TNF-α (100 ng per ml, 10 ng per ml, 1 ng per ml), IFN-α (1000 U per ml, 100 U per ml, 10 U per ml), IFN-γ (1000 U per ml, 100 U per ml, 10 U per ml), EGF (1000 ng per ml, 100 ng per ml, 10 ng per ml), bFGF (100 ng per ml, 10 ng per ml, 1 ng per ml), VEGF (1000 ng per ml, 100 ng per ml, 10 ng per ml), HGF (100 ng per ml, 10 ng per ml, 1 ng per ml), KGF (100 ng per ml, 10 ng per ml, 1 ng per ml), TGF-β1 (100 ng per ml, 10 ng per ml, 1 ng per ml), and IGF-1 (1000 ng per ml, 100 ng per ml, 10 ng per ml) for 72 h. Then, the culture supernatants were analyzed for leptin by ELISA. The data were expressed as the mean of triplicate cultures. Standard deviations of all the data were less than 5%. Here we present the representative data of three different experiments using FPC line 3306. Similar data were also obtained from the experiments using FPC lines 3301 and 3302.

These data suggest that leptin stimulates FPC in an autocrine fashion. So far, however, there are no reports of abnormal hair growth in either leptin-deficient (ob/ob) or leptin-receptor-deficient (db/db) mice. Recently, Frank *et al* (2000) demonstrated the mitogenic stimulus exerted by leptin on human keratinocytes. As most of the growth factors or cytokines that affect keratinocyte growth have been demonstrated to either stimulate or inhibit hair growth, it is still conceivable that leptin affects hair growth (Danilenko *et al*, 1996). Interestingly, when we examined the effects of growth factors or cytokines on the leptin production by FPC, those growth factors that are reported to stimulate hair growth, such as VEGF (Kozlowska *et al*, 1998), KGF (Rosenquist and Martin, 1996), HGF (Shimaoka *et al*, 1995), or IGF-1 (Little *et al*, 1994, 1996), did not suppress leptin production, whereas those reported to suppress hair growth, EGF (Moore *et al*, 1983; Philpott *et al*, 1994), bFGF (du Cros, 1993), and TGF-β1 (Sellheyer *et al*, 1993), significantly suppressed it. In addition, most proinflammatory cytokines and representative Th1 and Th2 cytokines significantly suppressed the production of leptin. These findings agree well with the fact that cytokines such as IL-1, TNF-α, and IFN-γ suppress hair growth (Cheng *et al*, 1992; Harmon and Nevins, 1993; Groves *et al*, 1995; Carroll *et al*, 1997). These data again strongly suggest a role for leptin in hair growth. We are currently investigating the effects of leptin on isolated follicles grown *in vitro*.

In this study, we examined the *in vitro* effects of various cytokines or growth factors on the production of leptin by FPC. Using adipocyte cell lines, the effects of TNF-α or TGF-β1 on the production of leptin have been examined. These studies demonstrated the suppressive effects of these two cytokines on the production of leptin by cultured adipocytes as seen in our observations using FPC (Gottschling-Zeller *et al*, 1999; Fawcett *et al*, 2000; Zhang *et al*, 2000).

In our study, leptin was detected in the epithelial cells of the inner root sheath and hair matrix of the hair bulb other than FPC. As we could not culture the inner root sheath or hair matrix *in vitro*, we did not further confirm their production of leptin. The hair follicle consists of upper and lower segments, both of which have two parts: an infundibulum and an isthmus for the former, and a stem and a bulb for the latter (Moschella and Hurley, 1992). Adamson's fringe is a well-known marker to separate the lower segment of the hair follicle into the stem and the bulb. Above this line, nucleated viable epithelium of the hair matrix becomes anucleate cornifying epithelium, Huxley's layer loses its trichohyaline granules, and hair tends to retract from the adjacent viable epithelial cells as an artifact. **Figure 5**(***a***, ***e***) demonstrates that hair follicles, in which hair does not retract from the adjacent epithelial cells, already lose the expression of leptin. This finding suggests that leptin expression is halted below Adamson's fringe. The hair bulb is further separated by the B-fringe and ''critical line'' into the upper keratogenous zone, the middle supramatrical zone, and the lower matrical zone. **Figure 5**(***a***, ***e***) clearly shows that the expression of leptin extends upward beyond the critical line that marks the site where the diameter of the bulb and papilla is greatest. Therefore, the upper limit of the expression of leptin is localized in the keratogenous zone, probably somewhere around the B-fringe marked by the loss of trichohyaline granules from Henle's layer.

Apart from the possible effects on hair growth, it is also possible that leptin plays a role in the regulation of the immune response or inflammation around hair follicles. Recently it has been reported that human hair follicle cells can be induced to grow in an incompatible host of the opposite sex (Reynolds *et al*, 1999). This finding suggests that the lowermost tissue of hair follicles is among a restricted group of immunoprivileged tissues. Recent studies using ob/ob or db/db mice (Yang *et al*, 1997; Faggioni *et al*, 1999; Takahashi *et al*, 1999) have demonstrated that the lack of leptin signal enhances their sensitivity to LPS, TNF-α, and IL-1. The significant production of leptin by hair bulbs may downregulate their sensitivity to these proinflammatory stimuli. These phenomena may be related to the characteristics of the lowermost tissue of hair follicles as an immunoprivileged tissue. So far, however, as most of the studies on the effects of leptin on immunity or inflammation have been conducted *in vivo*, we lack information on the *in vitro* immunologic effects of leptin on various types of cells, including keratinocytes or FPC.

Finally, our study demonstrated that, in addition to immunohistochemical evidence *in vivo*, FPC isolated from human scalp hair definitely produced leptin *in vitro*. So far, the characteristics of FPC that can be used for identifying them *in vitro* are so limited that their morphology and high expression of α-smooth-muscle actin are commonly used. Our observations provide additional information that will be useful for the identification of FPC.

This study was supported in part by grant 12670803 from the Ministry of Education, Science, and Culture of Japan, by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of the Japanese Government, and by the Cell Science Research Foundation.

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